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mRNA: Fulfilling the Promise of Gene Therapy

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In vitro–transcribed mRNA (IVT mRNA) is emerging as a new class of drug that has the potential to play a role in gene therapy that once was envisioned for DNA.[1] Although first described as a therapeutic in 1992,[2] IVT mRNA's immunogenicity prevented its development for protein replacement therapies. However, this problem was recently solved by the introduction of modified nucleosides into the IVT mRNA.[3] In this issue of *Molecular Therapy*, Thess and colleagues report an alternative method for generating nonimmunogenic IVT mRNA that avoids the use of modified nucleosides.[4] They demonstrate that sequence engineering of the mRNA and purifying it with high performance liquid chromatography are sufficient to avoid immune activation and to achieve high levels of translation of the encoded protein both *in vitro* and *in vivo*.

In previous studies using IVT mRNA in which all uridines were exchanged for pseudouridines—the most common naturally occurring modified nucleoside—the mRNA was found to be very efficiently translated and nonimmunogenic.[5] Here, Thess and colleagues opted to alter the sequence composition of the mRNA by incorporating the most GC-rich codon for each amino acid. When such sequence-engineered mRNAs encoding firefly luciferase and erythropoietin (EPO) were transfected into cells, more protein was produced from the mRNA generated using conventional nucleotides compared to modified ones. The only condition in which the pseudouridine-modified IVT mRNA was superior was when the IVT mRNA contained the native GC-poor coding sequence.

Using EPO-based hematopoiesis as an animal model system, they demonstrated that injection of mice with 1 µg of sequence-engineered mRNA containing uridines increases serum EPO levels and hematocrits to a higher level than the corresponding pseudouridine-containing mRNA. Even repeated injections of 10 µg of sequence-engineered mRNA (made with unmodified nucleotides) was found to be nonimmunogenic, as assessed by measuring inflammatory cytokines, which remained at baseline levels following treatment. Most importantly, when pigs and macaques were injected with lipid nanoparticle-formulated, sequence-engineered EPO mRNA, the authors observed significant increases of reticulocyte numbers and hematocrits. Thus, IVT mRNA could achieve a physiologically relevant parameter in large animals, thus opening potential opportunities to expand the therapeutic application of IVT mRNA to treat other diseases.

However, several important experiments remain. First, a side-by-side comparison of the sequence-engineered (GC-rich), uridine-containing mRNA vs. the native (GC-poor) mRNA containing pseudouridine should be performed *in vivo*. It is curious that in earlier work,[6] injection of mice with 0.1 µg of pseudouridine-containing EPO mRNA gave rise to EPO levels and reticulocyte values that are similar to what was achieved in the current study using 10 times more GC-rich EPO mRNA.[4] However, the most critical experiment is to unequivocally demonstrate a lack of immunogenicity of the codon-optimized sequences. Although Thess and colleagues reported no induction of tumor necrosis factor-α (TNF-α) and interleukin-6, these cytokines are not produced at sufficient levels when RNA is formulated with TransIT, a commercially available polymer. It has been demonstrated that whereas lipid-formulated RNA induces TNF-α, TransIT-formulated RNA primarily induces interferon-α (IFN-α).[7] It has been reported that mice injected with a positive control immunogenic RNA formulated in TransIT produce only IFN-α and not TNF-α or interleukin-6 (ref. 6). Unfortunately, IFN-α was not measured in any of the experiments. The positive control RNA that induced all of the measured inflammatory cytokines was unformulated (naked) and injected intramuscularly, whereas the test IVT mRNA was TransIT-formulated and injected intraperitoneally.

Future experiments will determine whether the sequence-engineered RNA is nonimmunogenic or that it retains partial immunogenicity. Nevertheless, it raises an interesting question: why are GC-rich sequences less immunogenic? They are prone to form stable double-stranded structures and thus would be expected to be more immunogenic. One explanation could be that increasing the GC content decreases the uridine (U) content of mRNA. Since U-rich RNA sequences are known activators of several RNA sensors, including Toll-like receptor 7 (ref. 8) and 8 (ref. 9), and RIG-I (ref. 10), it is possible that lowering the U content greatly reduces the immunogenicity of the RNA. It is also conceivable that some pathogens exploit this strategy to avoid the mammalian immune system. For example,

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the extremely high GC and therefore low U content of the herpesvirus family and mycobacterial genomes might be critical for these pathogens to avoid activation of the immune system.

Thus, based on accumulated evidence, it seems that to generate an optimal IVT mRNA for protein therapy, one needs to reduce or completely eliminate its U content without interfering with its translatability. This has been accomplished by (i) replacing all the uridines with pseudouridines, thus rendering the RNA nonimmunogenic;[6] (ii) replacing 25% of the uridines with 2-thiouridines, resulting in an mRNA with some residual immunogenicity;[11] or (iii) increasing the length of the poly(A) tail so as to reduce the relative U content or shielding the uridines in the RNA sequence and generating RNA with reduced immune potential.[12] Now, we can add a fourth strategy that involves modifying the codons to render the mRNA GC-rich, thus eliminating as many uridines as possible leading to reduced or abolished immunogenicity. Obviously, the level of U content will always depend on the amino acid content of the coding sequence, as half of the codons, including the start and the stop codons, must incorporate uridines.

Considering the enormous potential that IVT mRNA holds, we are looking forward to learning about exciting results as IVT mRNA enters clinical trials for protein therapy.

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Self-Destruct Genetic Switch to Safeguard iPS Cells

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Suicide gene approaches allow the conditional elimination of gene-modified cells and have been applied in cell therapy clinical trials. One such system, based on inducible apoptosis-mediated cell killing by caspase-9, which is activated by a nontoxic chemical inducer of dimerization (CID), has now been successfully applied in the context of induced pluripotent stem (iPS) cells.[1] The authors of the study engineered human iPS cells to express inducible caspase-9 (iC9) using lentiviral vectors, and demonstrated rapid CID-dependent apoptotic cell death in the parental iPS cells, in mesenchymal stromal cells differentiated from the iPS cells *in vitro*, and in teratomas generated *in vivo* following pluripotent stem cell transplantation in mice. Such a system is of paramount importance for enhancing the safety profiles of iPS cell–based products by elimination of unwanted cells, such as contaminating pluripotent cells or their differentiated progeny with genomic/epigenomic abnormalities having the potential to form teratomas following transplantation.

Direct reprogramming of differentiated somatic cells by gene transfer of a small number of defined transcription factors has been shown to yield cells that are highly similar to embryonic stem (ES) cells with respect to gene expression, morphology, pluripotency, and capacity for *in vitro* differentiation.[2] Due to their plasticity and unlimited capacity for self-renewal,

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